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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Construction of TBSR4-deficient strains of *Shewanella***

First (Bachelor's) level of higher education

Specialty 162 "Biotechnology and Bioengineering"

Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Ren Ziyi

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Scientific supervisor Olena Okhmat, Ph.D., Assoc. Prof.

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SUMMARY

Ziyi Ren. Construction of TBSR4-deficient strains of *Shewanella* – Manuscript.

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This topic is the construction of the missing strain of TBSR4 of *Shewanella*. In today's global fossil energy shortage and fossil energy exploitation process will cause certain pollution to the environment, microbial fuel cell has become an important research direction. *Shewanella* is a gram-negative bacterium and a commonly used electrogenic bacterium for microbial fuel cells. Therefore, it has a unique advantage as a microbial fuel cell.

Shewanella TBSR4 is dependent on a gene on the TonB iron carrier receptor on the outer membrane. TonB is related to iron homeostasis, which can affect the synthesis of cytochrome c and thus affect microbial electricity production. In this study, the effect of TBSR4 single gene knockout on the synthesis of cytochrome c was verified. After single gene knockout of TBSR4, the content of cytochrome c in the deletion type of TBSR4 was detected to determine whether TBSR4 gene of TBSR4 was related to the synthesis of cytochrome c.

In this study, single gene knockout of TBSR4 was performed by using in-frame deletion mutation method. The main steps include: plasmid extraction, overlapping PCR amplification of TBSR4 fusion fragment, construction and transformation of recombinant suicide plasmid, and construction of deletion mutant.

Although iron is abundant in nature, access to iron is often a challenge for microorganisms because the element exists primarily in the aerobic environment in the form of extremely insoluble iron Fe^{3+}. To overcome this problem, microorganisms have evolved a variety of iron uptake strategies, one of which is a common strategy by secreting ferriderites, which are endogenously produced iron-chelate metabolites. Ferrifer-mediated iron transport is an alternative route of iron transport under iron-rich conditions and is essential under iron-deficient conditions.

In Gram-negative bacteria, the transport of iron-ferriferic complexes from extracellular to periplasmic is primarily dependent on TonB-dependent siderophore receptors on the outer membrane. TBSRs) and TonB-ExbB-ExbD energy transduction system across intima and periplasm. Most TBSRs have similar domains: a barrel-like structure formed by 22 β folds and a globular stopper domain with n-ends. The plug domain binds to the substrate outside the outer membrane. When the iron-ferriderite complex binds to TBSR, the TBSR-TONB interaction domain changes. The TonB complex provides the energy converted by the electrochemical proton dynamic potential and transports the ferriderite complex into the periplasm. Subsequently, the Fe-ferriferal complex crosses the intima via ABC transporter or permease. In the cytoplasm, the iron in the Fe-ferriferal complex is reduced by reductase and released. In addition, under certain conditions, the iron-ferriferic complex can also be degraded in the periplasm, and the subsequently released Fe^{3+} is reduced to Fe^{2+}. Therefore, the study of TBSRs is of great significance for iron transport and cytochrome c synthesis in *Shewanella*.

Through a series of manipulative experiments, the correct missing strains were obtained, and the results of colony PCR and AGAR gel electrophoresis were consistent with expectations.

Key words: Microbial Fuel Cell, Shewanella, In-frame deletion mutagenesis; TBSR4gene

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INTRODUCTION

The relevance of the topic is Construction of TBSR4-deficient strains of *Shewanella*。

The purpose of the is to delve deeper into the exact relationship between the specific gene TBSR4 in *Shewanella* and Cytochrome c synthesis, and based on this discovery, further shed light on the potential link between *Shewanella* and iron production capacity. First, we focus on the microbiome of *Shewanella*, a class of bacteria that can survive in a variety of environments and is particularly known for its unique respiratory mechanisms and electron transport chains. Among them, cytochrome c, as a key electron transport protein, plays a crucial role in the energy metabolism and substance conversion process of *Shewanella*. The TBSR4 gene, as the focus of our research, may be closely related to the synthesis and regulation of cytochrome c, according to preliminary studies.

In order to verify this hypothesis, we will use molecular biology and genetics methods to conduct an in-depth study of TBSR4 gene in *Shewanella*. Specifically, we will observe the effects of these operations on the synthesis and activity of cytochrome c through technical means such as gene knockout, gene overexpression and mutant construction, so as to clarify the specific role of TBSR4 gene in the process of cytochrome c synthesis. After verifying the relationship between TBSR4 gene and cytochrome c synthesis, we will further explore the link between *Shewanella* and iron production capacity. We speculate that cytochrome c, as a key component of the electron transport chain, may be involved in the iron reduction process of *Shewanella*, and TBSR4 gene may play a regulatory role in this process.

Therefore, we will construct the exact strain of *Shewanella* TBSR4 to lay a foundation for future research.

The object of the study is the TBSR4 gene of *Shewanella*.

Research methods is the method of in-box gene mutation was used to construct TBSR4-deficient strains of *Shewanella* .

The practical significance of the results obtained is improve the iron production capacity of *Shewanella* and develop microbial fuel cells more efficiently. Verifying the relationship between *Shewanella* TBSR4 and the synthesis of cytochrome c can improve the production capacity in a more detailed and effective way, and can improve the disadvantages of today's traditional energy more accurately. MFCs and microbial electrolysis cells (MECs) systems built by *Shewanella* can not only produce energy such as electricity and hydrogen, construct related nanomaterials, but also produce high value-added chemical products. It provides more research directions for sustainable energy development. *Shewanella* biofilm plays an important role in electron transport and material exchange in bacterial cells. The study of *Shewanella* biofilm is of great practical significance for the development of environmental remediation and energy production. Secondly, the coupling of *Shewanella* biofilm and Fe_2O_3 coated electrode to drive the electric Fenton reaction can effectively accelerate the reduction rate of iron, and the total iron loss is less than 10%, and the removal rate of phenol is close to 100%, which provides an effective strategy for improving the efficiency of the electric Fenton reaction, solving the problems of iron loss and the formation of iron-containing sludge.

The scientific novelty lies in the unique iron-producing capacity of *Shewanella*. Compared with traditional energy sources, *Shewanella* has its own unique advantages. *Shewanella* is not only a kind of clean energy but also widely used. Therefore, *Shewanella* has unique research value.

CHAPTER 1
LITERATURE REVIEW

1.1 Development of microbial fuel cells

In the global energy arena, traditional fossil fuels such as oil and gas have always played a central role, providing indispensable power for the world's industrial development, economic prosperity and People's Daily lives. However, just as a coin has two sides, the wide application of fossil energy also brings many problems that cannot be ignored. First of all, we must face the biggest pain point of fossil energy is the non-renewable nature. As global energy demand continues to grow, fossil energy reserves are dwindling at an alarming rate, creating great uncertainty about our future energy supply. Secondly, the process of extracting and using fossil fuels causes great harm to the environment. In the mining stage, a large amount of waste gas and wastewater are discharged into the environment, causing serious pollution to soil and water resources. In the process of transportation and use, the combustion of coal and other fossil fuels will produce a large number of harmful gases, such as sulfur dioxide, nitrogen oxides and particulate matter, etc. These pollutants will not only lead to environmental problems such as acid rain and photochemical smog, but also pose a great threat to human health. What's more, fossil fuel use is linked to health problems in both children and adults. Emissions of soot and nitrogen oxide compounds can lead to a decline in air quality, which can lead to respiratory diseases such as allergies and asthma. This is especially serious for children, whose respiratory systems are not fully developed and are more vulnerable to pollutants.

In this context, the development of clean energy is particularly urgent. Microbial fuel cell, as a kind of clean and efficient renewable energy, is getting more and more attention. It uses microorganisms as a catalyst to directly convert the chemical energy in organic matter into electricity, which not only realizes the recycling of resources, but also avoids the emission of harmful gases, and is environmentally friendly and pollution-free.

Looking forward to the future, with the continuous progress of science and technology and the continuous improvement of environmental awareness, we have reason to believe that microbial fuel cells and other clean energy will occupy an increasingly important position in the global energy structure, and make greater contributions to the sustainable development of human society.

Microbial fuel cells are special fuel cells that use biocatalysts instead of traditional metal catalysts [1], and use microorganisms to directly convert chemical energy in organic matter into electrical energy [2]. When oxygen is scarce, microbes are able to break down organic matter in the environment, releasing responsive electricity-producing material. For example, the released electrons can be transferred in microorganisms and the environment by electron transfer, forming an electric current. The proton is transferred to the cathode through the proton exchange membrane, and then the oxidizer (oxygen) gets electrons at the cathode and is reduced to combine with the proton to form water [3]. Therefore, microbial fuel cells have unparalleled advantages over traditional batteries. In addition, the microbial fuel cell does not rely on material combustion in the process of electricity generation, and the energy conversion efficiency is not limited by the Carnot cycle. In recent years, researchers have successively selected and obtained a variety of microorganisms with electrogenic activity, which greatly enriched the application scope of electrogenic materials for microbial fuel cells, and showed great development potential and application prospects for alleviating energy crisis and developing green energy industry [4].

1.2 Power generation mechanism of *Shewanella*

Shewanella is a class of facultative anaerobic gram-negative bacteria with unusual respiratory (electron acceptor reduction) diversity, which is of great research value in bioenergy and environmental bioreaction [5]. When the chemicals in *Shewanella* come into contact with metals or minerals, they can generate an electric current through the cell membrane [6], which means that *Shewanella* can be directly

used for microbial electricity generation and thus for biofuel cells. The electrons produced by respiration in the cell of the bacterium can be transferred to the outside of the cell through its characteristic extracellular electron transport chain, thus generating an electric current. In this electron transfer process, the protein that contains iron to help electrons transfer within and between proteins is cytochrome c protein. Therefore, for *Shewanella*, the iron content in the cell plays an important role in its life and metabolic processes.

In the anoxic environment, in order to survive and adapt to the environment, microorganisms cannot get iron without iron carriers in the natural environment. Therefore, the effect of iron carrier on bacterial physiology is very important, and not only the simple iron chelation. In *Shewanella*, endogenous iron carriers play a very important role in biological processes directly related to the availability of iron. Although iron is abundant in nature, it is still difficult for microorganisms to obtain iron, because this element is mainly in the form of extremely insoluble Fe^{3+} in aerobic environments. To overcome this difficulty, microorganisms evolved a variety of iron uptake strategies[7]. One of the most common strategies is through the secretion of ferriderite, an iron-chelate metabolite that is produced endogenously. When iron content is relatively abundant, ferrifer-mediated iron transport is an alternative route of iron transport, but it is necessary under iron deficiency conditions. In G-bacteria, iron transport is mainly dependent on TonB iron-carrier associated receptors (TBSRs) on the outer membrane and TONB-EXBB-EXBD energy-related signal transduction system across the cell membrane and cytoplasm [8]. The transport of Fe-ferriferal complex from extracellular to periplasmic is mainly dependent on the fact that TBSRs genes in the outer membrane mostly have similar domains, namely 22-β folded barrel structure and n-terminal globular plug domain. The plug domain binds the substrate outside the cell membrane. When the iron-ferriderite complex binds to TBSR, the domain of the interaction between TBSR and TonB changes [9], and the TonB complex is used to provide energy converted by the electrochemical proton dynamic potential to transport the iron-ferriderite complex into the periplasm [10].

Therefore, the study of TBSRs is of great significance for iron transport and cytochrome C synthesis in *Shewanella*.

In addition to participating in iron intake, these ferriferous carriers also exhibit different biological activities that can influence other biological processes in some bacteria. For example, the loss of the ability to synthesize Putrebactin severely affects the manganese reduction capacity of *Shewanella*. In addition, Avaroferrin can significantly inhibit the movement of Vibrio [11]. In the natural environment, *Shewanella* and Vibrio often live in the same environment, so this iron carrier is considered to be a new means for *Shewanella* to adapt to the environment and gain competitive advantage [10] .Therefore, the research on TBSRs is of great significance.

1.3 Application prospect of *Shewanella*

Shewanella has respiratory diversity. *Shewanella* includes a group of facultative dissimilating metal-reducing bacteria that are widely distributed in water bodies and sedimentary environments that are generally characterized by chemical stratification. *Shewanella* contains a large amount of ferritin which contributes to the versatility of the respiratory system, especially ferrithionein and hemoglobin. Compared to E. coli, *Shewanella* has a relatively high iron content. Therefore, the iron demand of *Shewanella* has allowed *Shewanella* to evolve unique mechanisms to maintain iron balance in the body. As a facultative anaerobic electroactive microorganism, *Shewanella* has the advantages of clear genetic background and high electrochemical activity, and is one of the potential strains applied in bioelectrochemical reactions [12]. As a promising platform for renewable energy conversion and bioelectrochemical remediation, microbial electrochemical systems (MES) have attracted more and more attention. *Shewanella* is an allogeneic metal-reduction model bacterium with a variety of extracellular electron transfer (EET) strategies, which has become a popular microorganism in various MES devices for various practical applications and for the study of microbial EET mechanism. At the same time, with the continuous development of whole genome sequencing

technology, genes related to electricity production in bacteria will be discovered one after another, thus promoting the application of Shiva bacteria in MES.

Shewanella oneidensis MR-1 has the characteristics of electroactive biofilm and is commonly used as a carrier to achieve microbial electron transfer. Its formation process is very complex and is affected and regulated by many factors. It plays a very important role in enhancing bacterial environmental stress resistance and improving electron transfer efficiency [13].

Shewanella not only plays a direct role in energy production, but also shows unique research value in the field of materials science. In addition, *Shewanella* can effectively transform a range of heavy metals [14]. *Shewanella* mainly relies on biofilm for electron transfer and material exchange, and the research on the electroactive biofilm of *Shewanella* has very important theoretical guidance and practical significance in repairing the environment and generating energy [15]. Compared with traditional energy sources, *Shewanella* has its own unique advantages. *Shewanella* is not only a kind of clean energy but also widely used. Therefore, *Shewanella* has unique research value.

Conclusions to chapter 1

1. Microbial fuel cells, a cutting-edge field of science and technology, have attracted the attention of countless scholars since the beginning of their birth. From the initial theoretical framework construction, to the later experimental verification, and now the technological breakthrough, its development process can be described as twists and turns, but it is full of hope and opportunities. In the 21st century, with the rapid development of biotechnology and materials science, the performance of microbial fuel cells has been significantly improved, and it has become a bright star in the energy field.

2. Among the many microorganisms, *Shewanella* stands out for its unique power generation mechanism. The anaerobic bacteria, unlike other microorganisms, generate electricity by converting chemical energy from organic matter directly into

electricity through chemical changes in charged proteins inside the cell. This mechanism is not only efficient, but also environmentally friendly, providing a new direction for clean fuel cell research.

3. What makes the power generation mechanism of *Shewanella* so unique is closely related to the synthesis of its molecule DSFO+. This molecule plays a key role in the cells of the bacterium, effectively facilitating the transfer of electrons, allowing the bacterium to be more efficient in generating electricity. At the same time, the synthesis of DSFO+ also enhanced the adaptability of *Shewanella* different environments, so that it can stably generate current under different conditions.

4. In the field of agriculture, the application prospect of *Shewanella* is also broad. Due to its ability to generate electric current and provide the necessary energy support for the plant, it can improve the yield and quality of the crop. In addition, *Shewanella* can also improve the soil environment, increase soil fertility and water retention, and provide better environmental conditions for the growth of crops.

5. Looking forward to the future, with the continuous deepening of the study of *Shewanella*, its role in energy, environmental protection and other fields will be more significant. First of all, in the field of energy, *Shewanella* can be used as a new type of bioenergy, through large-scale breeding and the construction of power generation facilities, to provide clean and renewable energy for mankind. Secondly, in the field of environmental protection, *Shewanella* can be used as a bioreactor to treat various organic pollutants, such as wastewater, garbage, etc., to achieve the recycling of resources and environmental protection.

6. In short, as a unique microbial fuel cell research object, *Shewanella* has a broad development prospect and great potential. With the progress of science and technology and the pursuit of sustainable development, we believe that one day in the future, *Shewanella* will bring more surprises and changes to our lives and the environment.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

The purpose of this study is to provide a new idea for further understanding of the biological characteristics and iron production capacity of *Shewanella* TBSR4 by constructing an accurate strain of *Shewanella*, and to lay a foundation for subsequent research. The object of the study is the TBSR4 gene of *Shewanella*. Research methods is the method of in-box gene mutation was used to construct TBSR4-deficient strains of *Shewanella* .

2.1 Experimental material

2.1.1 Laboratory material

PCR product purification kit (Knogen); LB liquid medium; Exnase II (ClonExpress II, Vazyme); ddH2O; *CE II Buffer; LB plate (CM=20 μg/mL, DAP=0.3 mM, 0.3% D-Glucose); TaKaRa PrimerSTAR Max DNA Polymerase; pLP12Cm-UF/pLP12Cm-UR primer; LB-Cm-DAP; LB liquid medium without antibiotics; LB plate (0.4% L-arabinose, no antibiotics)

2.1.2 Chromosome locus of TBSR4 gene of *Shewanella*

ACCGTGAAGATATTCTCAATCTGCATTCCTTAAGCACTAAGCAATTG
GTTAATCTCGTGAATCGCTTAGAGGAAAGTCAGCTCGATAATTACACCGA
CATTCGTACCCTGATTGGCATCGTATTTGATGAAAATACCGTATGGGGCA
AGTTAACCATTATCGAACTTAAGATTTTGATTTATCTTGCACTTGGTGAAC
ATGAAGAAGCGCTAGAACTGGTCGGTGAATTCCTGCAATTTAACGACAAT
ACCGTGAAGCGCAATCTGTTTTACCAAGCAATGAGCGCGGTACTTGAAGT
GACGCTGGATGAAGATCTAGCTCTCGAAGACTTTAGCCATAACTTTACTC
GTATGTTTGGTGAGGAAGTGATGGCGCAAGTGGTTGGCAGCGTCACAGGT
AAAGTACGCTTTAGCGGCCTAACCAAGACCAGCATGCAACTCGAAGGGA
TTGAACCGCACTTAAGACTTATCGAAAGCTATAAGAAGCTGCATTCCGCC

CGCAAGGCCAACGCGGTTAAGTGCATTACTTTCTAATTTATTGATATATA
ATTAAAAAGCGAACATTTGTTCGCTTTTTTTATGCTCAATATACCTTGATA
TAATCTGCCGCCAAACTAACTTCCCGTAGTTGAGGGTTTTTAGCGGCAGT
GTTGGCTTCCCTTTCCCAATATTGCTGTGACTAATTATGCGAGTGTTATCT
GGGCAACGGATGCGGATACTGCGGGGCGACAGAGTAAGTGTATCTGATG
TTTGCTAAAAATGGCTTAAATGCGAAGGAGTTTTTCCTATTGAATATCGCC
CTCTTGCTGAGGGATGCGGCCTTATCTAAATCGATTTGCTGCTTCCTACGG
TGTTATAATCCTAGGGTTAGCTCGAGTGACAGAGTTGATTTCTAAGTTCTT
GCTCATCGGTAAAGCCTCGATATCCCTTGATTAATTCTCCTTTGGGATTTA
ACAGCAGTAAGATTGGCGTCGCGGGAATGCCACCCATCTCCTTTAGTAAA
GCAGGCGGAACGCGGTAGGCCTCAAAGGGAAAATTAAGCCTAAACAGGG
CGCGTTTTAAGGCCAAATTATCCCCATTGACCCCCAGCGCCACGGGCTGA
ATCGCTTGATTGCAGCTTTTATGCAGTTGCTTGAGTACGTTTGCCTGCTTA
ATACACCAAGGACATTCAGGTTCAAAGAACATCAACGCTGTGGATTGTCC
TTCTAGATGCGACAGA

2.1.3 Experimental primer design

Design according to the target gene as shown in Tab. 2.1.

Table 2.1 – Experimental primer design

Primer name	Primer sequence
TBSR4 MF1	CTGATGAGTAAAAGCTCGAGACCGTGAAGATATTCTCA
TBSR4 MR1	CTACGGGAAGTTAGTTTGGCGGCAGATTATATCAAGGT
TBSR4 MF2	ACCTTGATATAATCTGCCGCCAAACTAACTTCCCGTAG
TBSR4 MR2	GATATGTCGAGCTCGAATTCTCTGTCGCATCTAGAAGG
TBSR4 TF	CCAGTTAGGTGCATCGGCTT
TBSR4 TR	ACTGAGAAGCCAAACGCTCA

2.2 Research method

2.2.1 Overlap PCR

The Overlap PCR technique is a powerful tool in the field of molecular biology for precisely building or modifying DNA fragments. This technique is particularly useful for joining two DNA fragments with overlapping sequences to generate a completely new DNA molecule with a specific sequence. In the following, we will elaborate the principle and procedure of overlapping PCR technology.

The principle of overlapping PCR is based on the specific amplification ability of PCR technology [16]. First, two pairs of specific primers need to be designed, where each pair targets the upstream and downstream regions of the target DNA fragment. There is a complementary overlapping sequence between the two primers, allowing the DNA fragments produced by the two PCR amplification to overlap each other.

In the operation of overlapping PCR, the first step is the design of primers. This step is crucial because the specificity of the primer will directly affect the efficiency and accuracy of PCR amplification. The designed primers need to meet certain conditions, such as moderate length, GC content balance, no secondary structure, etc. Next is the preparation of PCR reaction system. This includes preparing template DNA (which can be genomic DNA, plasmid DNA, etc.), primers, dNTPs (four deoxyribonucleotides), DNA polymerase, and PCR buffers [17] .The proportions and concentrations of these components need to be adjusted according to the specific experimental conditions and objectives.

When the PCR reaction system has been carefully prepared and formulated, the next step is to accurately add these reaction fluids to the PCR reaction tube. These tubes are specially designed to withstand the high temperatures and cyclic changes generated during the PCR process, ensuring an efficient PCR reaction.

Next, we need to set up the proper procedure for the PCR reaction. The PCR procedure is set up according to the characteristics of the target DNA fragment and experimental needs, including pre-denaturation, denaturation, annealing and extension steps. The temperature and timing of these steps need to be precisely

controlled to ensure the specific amplification of DNA and the stability of the reaction. In the case of Overlap Extension PCR, two rounds of PCR amplification are usually required because two or more DNA fragments need to be joined. In the first round of PCR, we use external primers to amplify the upstream and downstream regions of the target DNA fragment respectively. These external primers are designed based on the target DNA sequence and are able to accurately identify and amplify the desired DNA fragments. After completing the first round of PCR, we need to mix the products of the two rounds of PCR as a template for the second round of PCR. In the second round of PCR, we use an internal primer for amplification. These internal primers are designed based on overlapping sequences of upstream and downstream DNA fragments and can guide the DNA polymerase to extend in the overlapping region, thereby connecting two DNA fragments. Because of the overlapping sequences between the internal primers, the second round of PCR was able to successfully amplify the complete DNA fragments connecting the upstream and downstream regions. This overlapping PCR technique has been widely used in many fields, such as gene cloning, gene recombination and gene editing.

Finally, we verified the size and quantity of PCR products by agarose gel electrophoresis. During electrophoresis, DNA fragments migrate through the gel depending on their size, forming different bands. The location and brightness of these bands can reflect the efficiency of PCR amplification and the purity and concentration of the product. By comparing the location and brightness of the bands, we can tell whether PCR amplification was successful and whether the product met expectations. The entire overlapping PCR process requires precise operation and strict control to ensure the accuracy and reliability of the experiment. Through reasonable experimental design and strict experimental operation, we can successfully use overlapping PCR technology to link and amplify target DNA fragments, which provides strong support for the subsequent genetic engineering and molecular biology research.

In conclusion, the overlapping PCR technique is an efficient and accurate method for DNA construction and modification [18]. Through rational design of primers and optimization of PCR reaction conditions, precise connection and modification of target DNA fragments can be achieved, which provides an important material basis for subsequent genetic engineering and protein expression experiments.

2.2.2 Plasmid construction

After successfully obtaining the desired fusion fragment, the next critical step is to insert this fusion fragment into the pLP12Cm suicide plasmid to construct a recombinant suicide plasmid containing the specific fusion plasmid. The pLP12Cm plasmid, as a derivative plasmid of the R plasmid, has a very wide range of host compatibility, which enables it to successfully replicate and express in a variety of bacteria. More importantly, the pLP12Cm plasmid is capable of conjugation transfer, meaning that it is able to efficiently transfer the target gene into the recipient cell.

Suicide plasmids are called "suicide" because their replication relies on a special protein that is not normally present in wild-type bacteria. Therefore, when this suicide plasmid is introduced into bacteria, there are two situations: if the bacteria does not have this special protein, the plasmid will not be able to replicate and will eventually be eliminated by the enzyme system within the cell; The second is that if this special protein is present in the bacteria, the plasmid can replicate successfully, and it is possible to integrate with the chromosome and replicate with the chromosome.

In this experiment, we inserted DNA fragments containing gene deletions into the pLP12Cm plasmid. This step is critical because it determines where the suicide plasmid integrates in the bacteria. By missing specific sequences at both ends of the gene, we can precisely control where the suicide plasmid inserts on the chromosome, enabling targeted gene editing.

In addition, the pLP12Cm plasmid also has a selective marker, which allows us to easily screen out bacteria containing the recombinant plasmid[19]. This selective marker is usually an antibiotic resistance gene, and only bacteria that successfully integrate the pLP12Cm plasmid can grow on a culture medium containing the corresponding antibiotic. This screening method is not only easy to operate, but also has high accuracy, which greatly reduces the workload of follow-up experiments.

To sum up, insertion of fusion fragments into pLP12Cm suicide plasmid requires precise control of experimental conditions to ensure correct insertion of fusion fragments and stable existence of suicide plasmid. Through this method, we can effectively perform gene editing on bacteria and provide strong support for subsequent biological research.

2.2.3 The suicidal plasmid pLP12Cm was transformed into receptive cell DH5α

Competent cells are a crucial concept in biological research, especially in the fields of genetic engineering and molecular biology. These cells are bacterial cells that have undergone a specific physical or chemical treatment, such as electric shock or $CaCl_2$ treatment, to temporarily enhance the permeability of their cell membranes. This special permeability state ensures that foreign DNA molecules can efficiently enter the cell, which provides the possibility for subsequent gene manipulation and genetic transformation [20].

DH5α is a widely used strain of Escherichia coli that is favored for its ease of culture and transformation. Through a series of carefully designed experimental steps, the researchers were able to induce DH5α cells to enter a receptive state. This process usually involves physical methods, such as electric shock, or chemical methods, such as $CaCl_2$ treatment. These treatments can temporarily change the stability of the cell membrane, making it more receptive to foreign DNA.

Specifically, $CaCl_2$ treatment is a commonly used method of chemical induction. When E. coli cells are exposed to a solution containing $CaCl_2$ at a low

temperature, the permeability of the cell membrane can be changed. This change allows DNA molecules to enter the cell through small holes in the cell membrane. The law of shock is to shock the cell membrane with a high voltage, causing it to briefly form small holes, allowing DNA molecules to enter.

Once cells are coaxed into a receptive state, they can be used to transform DNA or plasmids. In this process, researchers mix foreign DNA or plasmids with receptive cells and culture them under the right conditions. Due to the high permeability of receptive cells, these foreign DNA or plasmids can easily enter the cell and integrate into the genome of the cell. In this way, researchers can achieve precise editing and modification of bacterial genomes, providing a powerful tool for biotechnology and medical research.

2.2.4 Colony PCR was performed after culture

Colony PCR is an efficient and straightforward molecular biology technique that allows researchers to perform PCR amplification directly on a single colony without the cumbersome steps of extracting DNA from the bacterial genome[21]. The advantage of this method lies in its simplicity and rapidity, which brings great convenience for microbiology experiments. In colony PCR, the researcher selects a colony containing the target plasmid and directly uses it as a template for PCR amplification. Because the bacteria in these colonies usually already carry plasmids, and the plasmids often carry specific universal prim-binding sites, these universal primers can be used for PCR amplification. To perform colony PCR, a single colony is first picked with a sterile tool and transferred into a test tube containing the PCR reaction mixture. This reaction mixture contains all the components needed for PCR, such as primers, dNTPs, DNA polymerases, and buffers. Then, the test tube is placed in the PCR apparatus for amplification reaction. During PCR amplification, DNA polymerase replicates along the template DNA strand under the guidance of primers. Since the bacteria in the colony already carry the target plasmid, the PCR product will contain a fragment of DNA that corresponds to a specific sequence on the

plasmid. The length and sequence of these fragments can be detected and analyzed by methods such as electrophoresis. The result of colony PCR can directly reflect whether the target gene has been successfully transcribed into the bacteria. If a fragment of DNA appears in the PCR product as expected, it indicates that the target gene has been successfully integrated into the plasmid and is able to be transcribed and expressed in the bacteria. This provides an important basis for subsequent gene cloning, expression and functional analysis. In conclusion, colony PCR is a simple, rapid and effective microbial experimental method, which can directly amplify DNA fragments from a single colony, providing a strong support for genetic engineering and molecular biology research.

Before performing colony PCR experiments, researchers first need to carefully select a clearly visible monoclonal colony with neat edges from a petri dish. This step is crucial because monoclonal colonies ensure the specificity of the PCR reaction and avoid the confusion of experimental results caused by polyclonal mixing. Next, the monoclonal colony was inoculated into a PCR tube containing the PCR reaction solution in preparation for PCR amplification experiments. The predenaturation and denaturation steps of PCR reaction are the key steps in the experiment. At this stage, the bacteria in the reaction tube are rapidly cracked under the action of high temperatures, exposing the DNA inside the cell that was originally protected by the membrane. This process provides conditions for subsequent binding of PCR primers to DNA templates. In PCR amplification experiments, the use of specific primers is crucial to the success of the experiment. These primers are designed according to the target gene sequence and are able to precisely pair with specific regions on the DNA template. In addition, other components of the 25 μL PCR reaction system, such as dNTPs, DNA polymerase and buffer, also play an integral role. Under the precise control of PCR apparatus, these components work together with primers to complete the replication and amplification of DNA. After PCR amplification, the PCR products were analyzed by AGAR gel electrophoresis. This step visually displays the size and quantity of PCR products. By comparing with the expected results, we can judge

whether the PCR amplification is successful and whether the target gene is successfully transcribed into the bacteria. If the colony PCR detection product is positive, that is, the size of the amplified product is consistent with the expectation, it indicates that the target gene has been successfully integrated into the bacterial DNA, and subsequent experiments can be carried out. For positive colonies, purification culture is required to obtain sufficient number of bacteria. Then, plasmid pLP12Cm-TBSR4 was extracted from the positive bacteria using a plasmid extraction kit. This step requires attention to the normalization and accuracy of the operation to ensure that the extracted plasmid is of high purity and good integrity. Finally, the purified pLP12Cm-TBSR4 plasmid was introduced into Escherichia coli β2163 by electrotransformation. Electrotransformation technology is an efficient method of introducing foreign DNA, which uses high electric field pulse to change the permeability of cell membrane instantaneously, so that foreign DNA can enter the cell smoothly. In the process of electric conversion, parameters such as electric field intensity, pulse time and cell density need to be strictly controlled to ensure the conversion efficiency and quality [22]. After the electrotransformation, recombinant Escherichia coli β2163 containing the target gene can be obtained through screening and identification, which provides a basis for subsequent experiments and applications.

2.2.5 The transformed Escherichia coli β2163 was mixed with *Shewanella* after colony PCR

In microbiology research, the co-culture conjugation transfer technique is a powerful tool for transferring genetic material between different bacteria. In this particular experiment, we used this technique to transfer the recombinant plasmid pLP12Cm-TBSR4 from the donor to the recipient, specifically *Shewanella*. This transfer process requires not only precise experimental manipulation, but also a deep understanding of microbial genetics and molecular biology. Firstly, the donor bacteria containing the recombinant plasmid pLP12Cm-TBSR4 and the recipient

bacterium *Shewanella* were placed in the same culture environment through co-culture. Under the right conditions, such as specific temperature, humidity and nutrient composition, the two bacterial bodies come into contact with each other, thus achieving plasmid transfer. To screen for *Shewanella* that successfully metastasizes and inserts into specific locations on chromosomes, we take advantage of the characteristics of defective and resistance genes. These characteristics allow us to distinguish which strains have successfully received the recombinant plasmid and integrated it onto the chromosome. During the screening process, a plate containing a specific antibiotic is used to exclude strains that have failed to receive the plasmid because these strains are not resistant to antibiotics. Next, to further verify the screening results, colony PCR was used. From the selected insertion strains, monoclonal colonies were selected and diluted on LB plates containing L-arabinose. This step takes advantage of specific genes carried on the plasmid that make the bacteria sensitive to L-arabinose. Thus, only recombinant strains that successfully lost the pLP12Cm plasmid (or at least the part containing the gene) were able to grow normally on plates containing L-arabinose.

Finally, colony PCR was used to verify the missing mutant strains. By designing specific primers, we were able to amplify DNA fragments from these strains that matched expectations. The length and sequence information of these fragments will give us important clues as to whether the plasmid has successfully inserted itself into a specific location on the chromosome. If the PCR results are as expected, then we can confirm that we have successfully obtained recombinant *Shewanella* without pLP12Cm plasmid and can conduct subsequent experiments and studies. The whole process requires not only rigorous experimental operation, but also a deep understanding of microbial genetics and molecular biology. Through this method, we can effectively transfer and express foreign genes between microorganisms, which provides strong support for the research of genetic engineering and biotechnology[23].

Conclusions to chapter 2

1. Overlapping PCR technique: This technique is used to connect the upstream and downstream homologous arms of the missing fragment of the target, and by designing specific primers, PCR amplification is performed in both positive and negative directions to produce overlapping DNA fragments.

2. Construction of suicide plasmid: The fusion fragment obtained by overlapping PCR was inserted into the pLP12Cm suicide plasmid to construct the recombinant suicide plasmid. pLP12Cm is a plasmid derived from the R plasmid, capable of conjugating and transferring the target gene and unable to replicate in wild-type bacteria, thus ensuring that the target gene can be integrated onto the chromosome.

3. Receptive cell transformation: The suicidal plasmid pLP12Cm was transformed into Escherichia coli DH5α receptive cells, and the high permeability of receptive cells was used to allow foreign DNA to enter.

4. Colony PCR detection: PCR amplification is performed directly from the cultured colonies, and the products are analyzed by AGAR gel electrophoresis to determine whether the target genes are successfully transcribed into the bacteria.

5. Electrotransformation and mixed culture: The verified plasmids were introduced into Escherichia coli β2163 by electrotransformation technology, and then the recombinant plasmids were transferred to the recipient bacterium *Shewanella* by co-culture conjugative transfer technology. The characteristics of the defective type and resistance genes were used to screen, and the gene deletion strains of *Shewanella* were further verified by colony PCR.

CHAPTER 3
EXPERIMENTAL PART

3.1 Experimental principle

The TBSR4 gene of *Shewanella* was knocked out by the method of in-frame deletion mutation. First, Primer software was used to design specific primers for deleting the TBSR4 gene in *Shewanella*. The two DNA fragments were fused by overlapping PCR with two pairs of primers. Then, the PCR amplification product was attached to a cloning vector using restriction enzyme digestion, and the suicide plasmid was prepared. Finally, the obtained recombinant plasmid was transformed into the target cell by means of electrical transformation for screening and identification [24].

In-frame deletion mutation technique plays a crucial role in modern molecular biology research, and its advantages are significant and unique. First, this technique can subtly circumvent the problems that traditional gene inactivation methods may bring. Traditional methods of gene inactivation tend to block the normal function of genes by inserting or deleting key parts of the gene sequence, which can leave distinct genetic markers in the genome. However, the in-box deletion mutation technique is able to accurately delete a specific sequence in a gene without introducing any exogenous genetic markers, thus achieving functional inactivation of the target gene. More importantly, in-frame deletion mutations do not trigger early termination codons or cause the read frame to shift. This means that although part of the sequence of the target gene is deleted, the remaining gene sequence can still maintain its original reading framework, ensuring the normal process of protein translation. This characteristic makes in-box deletion mutations a powerful tool for studying gene function and protein expression.

In the experiment, with the successful construction of TBSR4 gene missing mutant strains of *Shewanella*, we can further study the specific function of TBSR4 gene in the electricity generation process of *Shewanella*. *Shewanella* is a kind of

microorganisms with unique ability of generating electricity, which has important application value in the fields of bioelectrochemistry and microbial fuel cell. By constructing TBSR4 gene missing mutant strains, we can observe and compare the differences in electrical production performance between wild type strains and mutant strains, and reveal the mechanism of TBSR4 gene in the electrical production of Shewanella.

In addition, in-box deletion mutation technology can also be used to construct other gene deletion mutation strains, providing a powerful tool for the study of various biological problems. Through this method, we can more deeply understand the function of genes, regulatory mechanisms and interactions with other biomolecules, and provide new ideas and methods for the research of biomedicine, agriculture, environmental protection and other fields.

3.2 Experimental procedure

3.2.1 Genome extraction of *Shewanella*

The whole genome of *Shewanella* was extracted with the bacterial genome extraction kit produced by Tiangen. Then find the genetic sequence of *Shewanella* TBSR4 on the NCBI website. After finding the target sequence according to the target gene, the upstream and downstream homologous fragments of the target missing gene were designed as PCR amplification primers, which were TBSR4-MF1/ TBSR4-MR1 and TBSR4-MF2/ TBSR4-MR2 (hereinafter referred to as A and B fragments), respectively. Secondly, PCR amplification procedures were set as follows: Predenaturation 95℃, 10 min; Denaturation 95℃, 30 sec, annealing 56℃, 20 sec, extension 72℃, 1min, this procedure is 35 cycles; The fragment was extended at 72 ° C for 10 min. The enzyme used in this step was purchased from PrimerSTAR Max DNA Polymerase from Dalian Baobiological. PCR amplification products were recovered using PCR product recovery kit (Tiangen). The results of agarose gel electrophoresis are shown in Fig. 3.1.

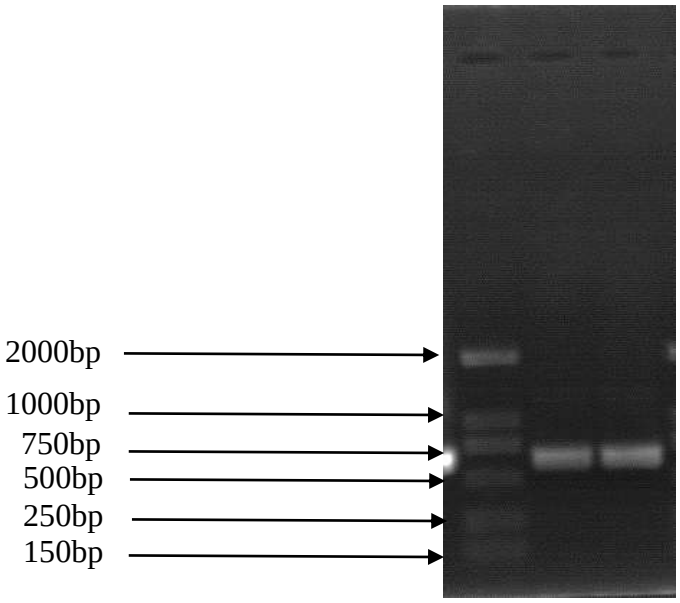

Figure 3.1 – Amplified fragment of TBSR4

Note: Lane 1: DL2000 DNA Marker; Lane 2: Segment A; Lane 3: Segment B

3.2.2 The TBSR4 fusion fragment was amplified by overlapping PCR

In this experiment, two pairs of specific primers were designed. The outer primer contained the end base sequence of the first DNA fragment (fragment A), and the inner primer contained the start base sequence of the second DNA fragment (fragment B), so that the end and start sequence of the two DNA fragments could overlap. In this step, the A and B fragments amplified and purified above were used as templates for overlapping PCR amplification. The specific procedure set the amplification cycle (Part 1) as follows: predenaturation 98℃, 1 minute; Denaturation 98℃, annealing 65℃ in 10 seconds, extension 72℃ in 20 seconds, and 7 cycles of the above steps for 1 minute. Amplification cycle (Part 2) : denaturation 98℃, 10 sec; Annealing at 56℃, 20 sec extended at 72℃ for 1 min, and the above operation was repeated for 35 cycles. Final elongation 72 ° C, 7 minutes. After overlapping PCR, the fusion A and B fragments were obtained, the length of which was 1218 bp. PCR amplification product recovery kit (Tiangen) was used to purify and recover the fusion DNA fragments. The results of agarose gel electrophoresis are shown in Fig. 3.2.

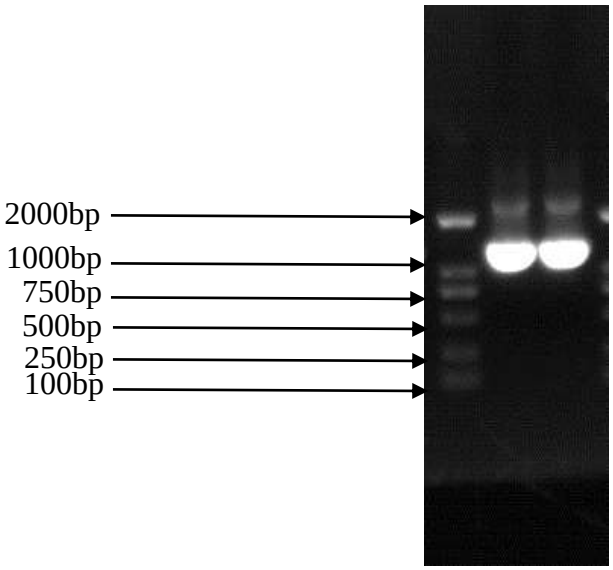

Figure 3.2 – Overlapping amplified fragments

Note: Lane 1: DL2000 DNA Marker; Lane 2, 3: Blending AB fragments

3.2.3 Plasmid extraction

Escherichia coli DH5α carrying pLP12Cm plasmid was activated and inoculated into LB liquid medium and cultured in a 180 × R shaker at 37℃ for 10 hours.

After the bacterial liquid was centrifuged, the bacterial inner plasmids were extracted according to the instructions of the plasmid extraction kit. The concentration and structure of the extracted plasmids were analyzed by agarose gel electrophoresis.

3.2.4 Construction and transformation of pLP12Cm-TBSR4 recombinant suicide plasmid

In the experiment of constructing pLP12cm-TBSR4 recombinant suicide plasmid, the purified AB fragment was first connected to the suicide plasmid vector pLP12Cm [25], and the linking system was placed at 37℃ for 60 min for effective connection between the DNA fragment and the plasmid vector. Place the connection product on ice for 10 minutes. Then the recombinant plasmid was introduced into Escherichia coli receptor cell DH5α by electrotransformation according to the

previous research method. The transformed cells were coated on an LB plate containing chloramphenicol (20 μg/mL) and D-glucose (0.3%)[25]. The plates were incubated in a constant temperature incubator at 37 ° C for 14 hours. After that, the growing E. coli colonies were tested by colony PCR, and the colonies were verified by PCR amplification using pLP12Cm-UF/pLP12Cm-UR universal primers. The PCR amplification products were analyzed by agarose gel electrophoresis, and the recombinant cloned genes inserted by AB fragment were screened out. The positive cloned genes were purified and expanded for culture, and then plasmid pLP12Cm-TBSR4 was extracted from the positive colonies using a small medium plasmid extraction kit. The colony culture results are shown in Fig. 3.3.

Figure 3.3 – Transformation of E. coli DH5α plate by recombinant plasmid pLP12Cm-TBSR4

The purified plasmid pLP12Cm-TBSR4 was introduced into Escherichia coli β2163 receptive cells by electrotransformation, the cell suspension was transferred to the preheated resuscitation medium, and the transformed product was placed under appropriate conditions for recovery culture for 5 hours. Then, E. coli suspension was coated on LB plate containing chloramphenicol and D-glucose with a coating rod. TBSR4-MF1/TBSR4-MR1 was used as the primers to perform colony PCR verification for the bacterial colonies grown on the plate, and the PCR amplification

products were analyzed by agarose gel electrophoresis. The positive strains containing pLP12Cm-TBSR4 were selected. Then the positive colonies were striated and purified. The colony culture results are shown in Fig. 3.5. The results of agarose gel electrophoresis are shown in Fig. 3.4 and 3.6 respectively.

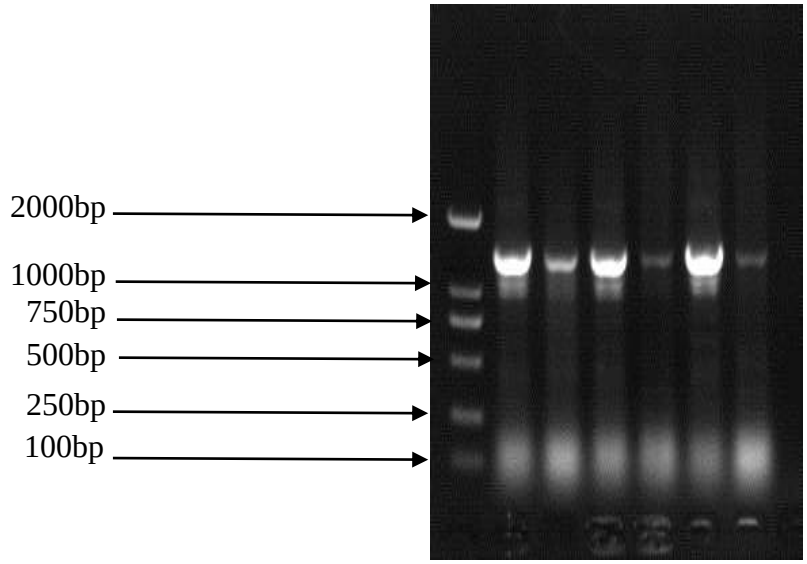

Figure 3.4 – pLP12Cm - TBSR4 plasmid detection

Note: lane 1: DL2000 DNA Marker; Lane 2-7: Positive recombinant clone

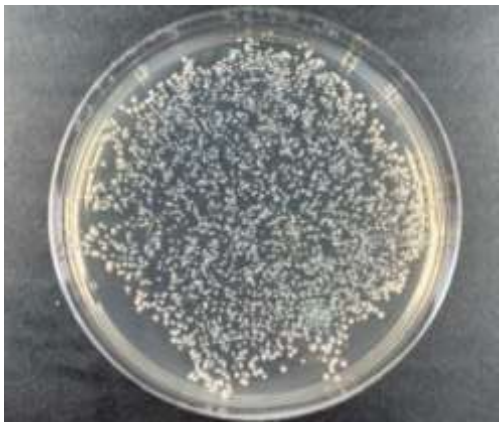

Figure 3.5 – Recombinant plasmid pLP12Cm-TBSR4 Electrotransβ2163 plate

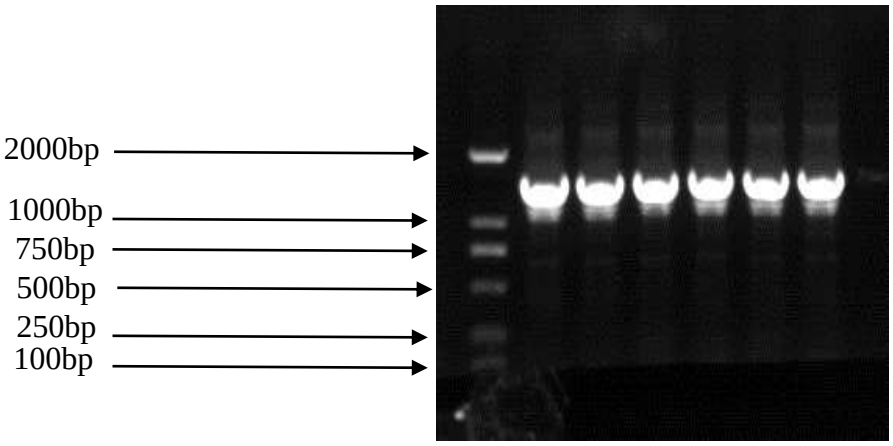

Figure 3.6 – Beta 2163 electrotransformation results colony PCR detection

Note: lane 1: DL2000 DNA Marker; Lane 2-7: Positive recombinant clone

3.2.5 Construct mutant strain

Escherichia coli β2163 (a donor with pLP12Cm-TBSR4 recombinant suicide plasmid) was inoculated in LB liquid medium containing chloramphenicol and diaminoheptanediic acid at 37℃ and cultured at 200 × R for 8 h. Escherichia coli β2163 was cultured in ordinary LB liquid medium at 37℃, 200 × R for 8 h. 200 microliters of cultured Escherichia coli β2163 donor bacteria and Escherichia coli β2163 were mixed in a sterile centrifuge tube and centrifuged (8000 × R, 10 min) to precipitate the bacteria. After the supernatant was discarded, the bacteria were re-suspended with LB liquid medium [25] and centrifuged a second time. Discard the supernatant after centrifugation. LB liquid medium was re-suspended, centrifuged for a third time, and the supernatant was removed again. 10μL LB liquid medium was added to the suspension mixture, and the suspension drops were added to the LB plate containing diaminopimelic acid (3 mM) and D-glucose (0.3%). The culture was incubated at 37℃ for 6 h. After suspension, 1 mL of LB liquid medium was used for dilution, and 100μL of diluted bacterial solution was applied on LB plate containing chloramphenicol (20μg/mL) and D-glucose (0.3%). Because E. coli β2163 belongs to the diaminoheptanediotrophic strain, it cannot grow on LB nutrient AGAR without D-glucose, while *Shewanella* mutants without the introduction of the missing plasmid cannot grow on LB nutrient AGAR containing chloramphenicol. When the

pLP12Cm-TBSR4 plasmid was genetically cross-exchanged with the *Shewanella* chromosome, the mutant *Shewanella* was ensured to grow on LB nutrient AGAR containing chloramphenicol (20μg/mL) and D-glucose (0.3%). The colony culture results are shown in Fig. 3.7.

Figure 3.7 – Recombinant plasmid pLP12Cm-TBSR4 integrated into a *Shewanella* plate

Several positive clones were selected from LB selection plates containing chloramphenicol (20μg/mL) and D-glucose (0.3%) and striated for purification. Since the exogenous plasmid pLP12Cm-TBSR4 contained sequences homologous to the target mutant, these homologous sequences were inserted into the chromosomes of the target mutant through homologous recombination, and then the colonies were selected after culture and colony PCR was performed for validation. The purified monoclonal liquid was selected and cultured, and the clones were detected by TBSR4-TF/pLP12Cm-UTR. The results of agarose gel electrophoresis showed that a specific amplified band with a size of 1436 bp was formed. The results of agarose gel electrophoresis are shown in Fig. 3.8.

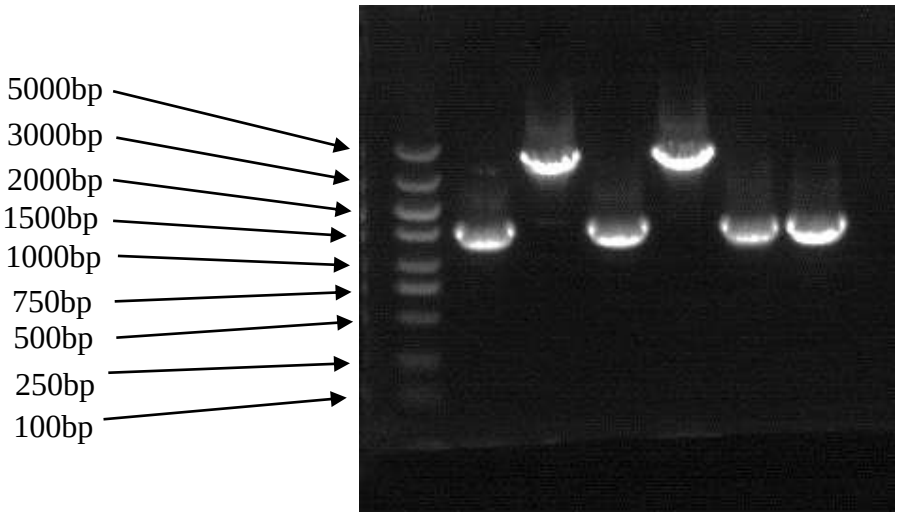

Figure 3.8 – TBSR4 insertion mutant detection

Note: lane 1: DL5000 DNA Marker; Lane 2-7: Positive recombinant clone

3.3.6 Deletion mutant construction

Firstly, positive clones were selected from the selected interstitial strains and added to LB liquid medium without antibiotics, which was oscillated in a constant temperature incubator at 37℃ for 10 hours, and then the bacterial solution was properly diluted to a concentration of 1.0×108 CFU/mL. The bacterial solution was evenly coated on LB nutrient AGAR containing L-arabinose but without chloramphenicol and cultured in a constant temperature incubator at 37℃ for 10 hours. A number of colonies were then selected and inoculated on a plate containing L-arabinose (LB-arabinose) and another containing chloramphenicol. And mark the colony locations on both plates. The two plates were simultaneously cultured in a constant temperature incubator at 37℃. Clonal colonies that could grow on LB-arabinose selective medium but could not grow on LB-chloramphenicol selective medium were selected. Then, TBSR4-TF/pLPCm12-UTR was used as specific primers for colony PCR amplification. It was confirmed that mutant clones could amplify the corresponding size of DNA bands, while wild-type clones could not amplify PCR products. The mutant clones were inoculated on LB plates containing L-Arabinose and continued to be cultured in a constant temperature incubator at 37℃. The colony culture results are shown in Fig. 3.9.

Figure 3.9 – *Shewanella* TBSR4 deletion mutant plate

Finally, positive clonal colonies were selected from LB plates and amplified using TBSR4-TF/TBSR4-TR consent primers. Then the PCR products were detected by agarose gel electrophoresis with wild type strains as the control. The correct deletion mutant clone in the gel electrophoresis result will produce a DNA fragment of 2252bp, in contrast, the wild-type strain does not have the deletion mutation, and the PCR product fragment is shorter, with a length of 1343bp. The results of agarose gel electrophoresis are shown in Fig. 3.10.

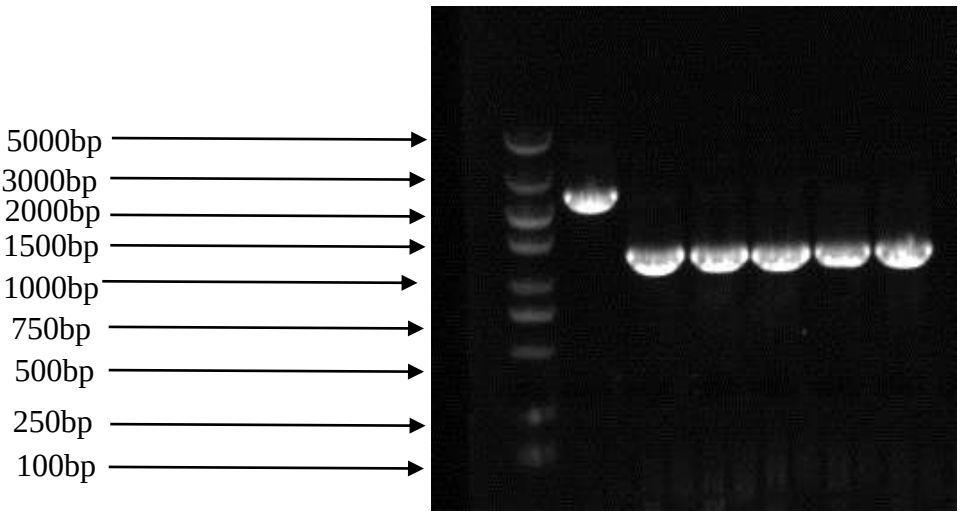

Figure 3.10 – Contrast agarose gel electrophoresis of TBSR4 deletion mutation

Note: lane 1: DL5000 DNA Marker; Lane 3-7: missing mutant; Lane 2: Wild type strain

3.4 Discussion of the results

3.4.1 Amplification of target deletion gene

Primers TBSR4-MF1/ TBSR4-MR1 and TBSR4-MF2/ TBSR4-MR2 were used to amplify the A and B fragments of the upstream and downstream homologous arms of *Shewanella* TBSR4. The glue running results showed that there was a clear and single band near 600 bp. The results of agarose gel electrophoresis of PCR amplification products are shown in Figure 3-1. Then, PCR product purification kit (Tiangen) was used to purify and recover the PCR amplification products.

3.4.2 Overlap PCR

Using amplified and purified fragments A and B as templates, overlap PCR was performed for amplification. The glue running result showed a clear and single strip at 1240bp, as shown in Figure 3-2 for the amplification diagram. The gene was amplified by PCR using PrimerSTAR Max DNA Polymerase. The results showed that the A and B fragments were successfully fused, and the fusion product with a length of 1218 bp was obtained. PCR product purification kit was used to purify the AB fusion fragment.

3.4.3 Conversion

The amplified and purified AB fusion gene was linked to pLP12Cm using Exnase II (ClonExpress II, Vazyme) ligase, ddH2O and 5*CE II Buffer. After that, the linking system was introduced into Escherichia coli receptor cells DH5α by means of electrical transformation. Then, positive colonies carrying the suicide plasmid pLP12Cm were selected and inoculated in LB liquid medium for overnight culture under appropriate conditions. Then it was coated on LB plate for culture. After culture, plasmid extraction could be carried out. After culture, the colonies were obviously clear to see 3-3, and the results were in line with expectations.

3.4.4 Colony PCR verification

Colony PCR verification was performed on Escherichia coli colonies. TBSR4-MF1/TBSR4-MR1 plasmid universal primers were used for colony PCR amplification, and the positive colonies were verified to contain target genes. The results (Figure 3-4) showed that the positive clone had a clear and single band at 1460 bp, which was consistent with the expected results.

3.4.5 Electrical Conversion

In the process of electrotransformation of purified pLP12Cm-TBSR4 plasmid into Escherichia coli β2163, because Escherichia coli β2163 belongs to diaminoheptanediotrophic strain, this strain cannot grow on LB nutrient AGAR without D-glucose. However, *Shewanella* mutants without the introduction of the missing plasmid could not grow on LB nutrient AGAR containing chloramphenicol. Therefore, only the pLP12Cm-TBSR4 plasmid can grow on the LB-selective plate when there is gene cross-exchange between the PLP12cm-TBSR4 plasmid and the Shiva chromosome. Positive colonies were purified by stripe inoculation on LB nutrient AGAR, and the colonies were clearly visible after culture, as shown in Figure 3-5. The results were in line with expectations, and colony PCR validation could be continued.

3.4.6 Agarose gel electrophoresis detection

The target strains of homologous recombinant were screened by colony PCR, and the positive clones of transformed Escherichia coli β2163 were detected by electrophoresis using TBSR4-TF and pLP12Cm-UTR as primers. The results of agar-gel electrophoresis were shown in Figure 3-6. The results showed that a clear and single amplification band of 1436bp was formed, and the electrophoretic results were consistent with the expectations.

3.4.7 Integrated Culture

The culture results were shown in Figure 3-7. LB liquid medium without chloramphenicol was cultured for 8 hours, and then the bacterial solution was evenly coated on LB nutrient AGAR with a coating rod. The transformed Escherichia coli β2163 positive clone was co-cultured with Shewanella, and the plasmid pLP12Cm-TBSR4 was integrated into *Shewanella* through conjugation, and the colony was clearly visible after culture.

3.4.8 Electrophoresis Detection

Select s *Shewanella* clones that can grow on LB-arabinose plates but cannot grow on LB-Cm (chloramphenicol containing) plates. Positive monoclonal strains were selected for liquid purification culture, and the colonies were analyzed by agarose gel electrophoresis using TBSR4-TF and pLPCm12-UTR specific primers. The results of gel electrophoresis (FIG. 3-8) showed that two positive bands of about 3245 bp and 1636bp were amplified by PCR.

3.4.9 Colony culture

After the deletion of plasmids through the second homologous recombination, some monoclonal colonies that could grow normally on LB-arabinose plate but could not survive on LB-Cm plate were selected for culture, and clear and obvious colonies were visible after culture, as shown in Figure 3-9.

3.4.10 Electrophoresis detection

TBSR4-TF and TBSR4-TR were used as primers for PCR amplification, and wild-type strains were used as negative controls. When the plasmid was missing, DNA bands with the size of 1343 bp could be amplified by PCR, while those with the size of 2252bp could be amplified by wild-type PCR. The rubber running results showed that strips of about 1300 bp were obtained for runway 3-7, and strips of about 2200 BP were obtained for runway 2. The rubber running results were shown in Figure 3-10, which was consistent with expectations.

Conclusions to chapter 3

1. Design overlapping PCR primers: First, based on the genome sequence of *Shewanella* TBSR4, design specific overlapping PCR primers that can cover the target deletion region.

2. Overlapping PCR amplification: Using designed primers, DNA fragments containing upstream and downstream sequences of the target deletion region are amplified by overlapping PCR technology. The purpose of overlapping PCR is to ensure the correct connection of the upstream and downstream sequences, forming a complete DNA fragment containing both sides of the missing region.

3. Constructing a suicide plasmid: The DNA fragment obtained by overlapping PCR is inserted into a suicide plasmid (such as pLP12Cm) to construct a recombinant suicide plasmid containing the target deletion region. The properties of suicide plasmids enable them to replicate themselves under certain conditions (such as in a specific bacterial host), but they are not stable in normal bacteria, ensuring that the target genes can be successfully integrated onto the chromosomes.

4. Transforming receptive cells: The constructed recombinant suicide plasmid is transformed into receptive cells (such as E. coli DH5α), so that the DNA fragment of the target deletion region can be stable and replicated in the cell.

5. Colony PCR validation: Monoclonal colonies are selected from transformed cells and colony PCR validation is performed to ensure that DNA fragments of the target deletion region have been successfully integrated into the chromosome.

6. Conjugative transfer: By conjugative transfer technique, suicide plasmids integrating DNA fragments of targeted deletion regions were transferred from E. coli DH5α to *Shewanella* TBSR4. During conjugation transfer, the suicide plasmid integrates onto the chromosomes of the bacterium, thereby achieving deletion of the target gene.

7. Screening and validation of missing strains: Appropriate screening markers (such as antibiotic resistance) are used to screen out *Shewanella* TBSR4 missing strains that successfully integrate the target deletion region, and further experiments are conducted to verify the phenotype and function of the missing strains.

CONCLUSIONS

1. The TBSR4 gene belongs to the TBSR family and is closely related to the synthesis of cytochrome c, a function of which is critical for the bacterium. To investigate this function, the TBSR4 gene of *Shewanella* was knocked out. In the knockout process, in-frame gene deletion method was mainly used. The A and B fragments were obtained by PCR amplification, and then colony PCR, agarose gel electrophoresis and DNA sequencing results showed that the coding region of TBSR4 gene was completely deleted and the upstream and downstream fragments were seamlessly connected.
2. The above results showed that the TBSR4 gene deletion mutant strain of *Shewanella* was constructed in the experiment. This strain will provide important material for the subsequent study of TBSR4 gene and cytochrome C synthesis of *Shewanella*.

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